Construction and Histopathological Characterization of Multiple Virulent Genes Mutant of *V. cholerae*: To Understand the Enteropathogenesis of Cholera

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Cholera is caused by toxigenic *V. cholerae* that secretes the cholera toxin (CT) encoded by *ctx* gene. Although CT is the major toxin, accessory toxins *rtx* gene encoding RTX toxin and *hap* gene that secretes hemagglutinin/protease (HA/P) have been shown to elicit mild diarrhoea and inflammatory reactions. The present study was designed to produce a *V. cholerae* O139 vaccine candidate having multiple virulent genes mutation and study their enteropathogenesis in animal model. The *ctx*, *rtx* and *hap* genes were mutated individually by allele replacement method and the vaccine candidate were named as VCUSM14P, VCUSM10P and VCUSM17P respectively. A multitoxin - deficient mutant (VCUSM22P) was created that have all 3 genes mutated. The mice colonization ability in all the above mentioned individual and multitoxin - deficient mutants were good and were 1 log lower that of wild type (WT) strain. In ileal loop assay, no fluid accumulation were seen in VCUSM14P and VCUSM22P which have the *ctx* gene mutated, while VCUSM10P and VCUSM17P showed more fluid accumulation as seen in the WT strain due to the presence of intact *ctx* gene. The histopathological studies, correlated with the fluid accumulation data wherein the tissue sections infected with VCUSM14P and VCUSM22P showed the presence of intact villi, intestinal gland with no damage in the submucosa, muscularis or serosal layer. But VCUSM10P and VCUSM17P caused sloughing of the villi, mild to moderate haemorrhage, congested blood vessel in submucosa and PMN in the lamina propria, as in tissue infected with WT strain. The histopathological features were further confirmed by immunohistochemical analysis. Thus it is clear that the mutation of only accessory toxins (RTX and HA/protease) caused only modest changes in virulence. While multitoxin - deficient mutant (VCUSM22P) are potent vaccine candidate which shows better colonization, low virulence with no enteropathogenic, cytopathic and haemolytic effects.

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***In-vitro* and *In-vivo* Studies on the Effect of Using Single and Combinations of Antibiotics on Different Phases of Bacterial Growth**

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Background: In our few working Sudanese microbiological laboratories, sensitivity testing for isolated organisms is centrations. Accordingly the antibiotics are judged whether they are effective (sensitive) or ineffective (resistant). In this test, the bacteria used are always in the stationary phase or declining phase. This situation does not answer several important questions.

Is it possible to overcome bacterial resistance by using different combinations of commonly used antibiotics? Do bacteria at different phases of growth, starting from lag phase to declining or death phase behave similarly in their sensitivity to various combinations of antibiotics? Are the effects of these combinations the same *in vivo* as *in vitro*?

The aim of the present study is to render available a clue about the use of combinations antibiotics so as to find out the most effective time of starting the treatment by antibiotics, at the beginning of onset of disease or latter when symptoms have clearly developed in both man and animals. This aim can be achieved through the following objectives:

1. To test the sensitivity of selected resistant Gram negative organisms to combinations of commonly used antibiotics in Sudan.
2. To compare the sensitivity of bacteria at different phases of growth to antibiotics when using single antibiotic or combinations of antibiotics.
3. To monitor *in vivo* the sensitivity of selected bacteria at different phases of growth to single and combinations of antibiotics, which were found to be effective *in vitro*.

Methods: Bacterial strains and susceptibility testing

Pasturella multocida local sensitive strain to most of the antibiotics which are commonly used in Sudan had been used in these studies.

Antibiotics Erythromycin and tetracycline each separately and in combination were used in these experiments.

In vitro studies (growth curve with antibiotics)

Ten-ml sterile bottles of nutrient broth were inoculated by a colony or part of a colony of *Pasteurella multocida*. The inoculum was emulsified carefully and the bottle was incubated at 37 °C for 18 hours. Eight bottles containing 99 ml Mueller Hinton Broth each were prepared and were inoculated with one ml from the overnight culture. The eight were labeled time zero, one hour, two hours, four hours, six hours, eight hours and twenty four hours. From the first bottle (at zero time) one ml was discarded and replaced by one ml normal saline containing the tested antibiotic of 1 MIC concentration (erythromycin = 2 μgm/ml, tetracycline = 4 μgm/ml and combination of erythromycin/tetracycline = 0.125/0.25 μgm/ml). The antibiotic was allowed to act for 15 minutes then the viable count was done. The other seven bottles were incubated at 37 °C for 1 hour, 2 hours, 6 hours, 8 hours and 24 hours respectively. After the inoculum time 1 ml was discarded and replaced by 1 ml normal saline containing the antibiotic of 1 MIC concentration. Then after 15 minutes viable count using Miles and Misra (1938) method was done.

The results of the viable count were compared with a standard curve for *Pasteurella multocida* (control) without the addition of any antibiotic.

In vivo studies.